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Biosynthesis and processing of legumin-like storage proteins in *Lupinus angustifolius* (lupin)

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Synthesis, secretion and post-translational proteolysis of the storage proteins in cotyledons of *Lupinus angustifolius* L. (lupin) have been examined *in vivo* and *in vitro* by using a combination of pulse–chase experiments with [^{3}H]- or [^{35}S]-labelled amino acids, subcellular fractionation and cell-free translation from poly(A)$^+$ (polyadenylylated) RNA or membrane-bound polyribosomes. Related polypeptides were identified by immunoprecipitation, separation on sodium dodecyl sulphate/polyacrylamide gels and fluorography. The synthesis and processing of two proteins were compared. Conglutin α, the 11 S protein, was found as a family of precursor polypeptides of M_r 68000–88000 when translated from poly(A)$^+$ RNA under conditions where signal segments were not cleaved, and M_r 64000–85000 both when sequestered into the endoplasmic reticulum and when accumulated in the protein bodies. Pulse–chase labelling showed that cotyledons from early stages of development were completely incapable of further proteolysis of these precursors. Nevertheless, in the same juvenile cotyledons, the precursors of the minor storage protein conglutin γ, two polypeptides with M_r 50000–51000, were proteolytically cleaved to mature subunits of M_r 32000 and 17000 within 2 h. Further cleavage of the precursors of conglutin α into families of mature subunits of M_r 21000–24000 and 42000–62000 was detected in more mature cotyledons. A model is proposed which suggests that the mature subunits are produced by a single proteolytic cleavage of each of the three major precursors of conglutin α and also suggests that a close similarity exists between these subunits and those of other legumin-like proteins. The enzyme responsible for this cleavage, which appears at a specific stage in the middle of cotyledonary development, seems to be an integral part of the programmed developmental sequence in these pods.

INTRODUCTION

Storage proteins of legumes are produced in developing cotyledons during a burst of protein synthesis. High levels of mRNA precursors, intermediates and accumulated products make these systems ideal models for protein synthesis (Higgins, 1984). Since, by definition, storage proteins accumulate within protein bodies, they may be regarded as secretory proteins. Study of the mechanism of their movement from the site of synthesis to the protein bodies may serve to further define intracellular transport (Chrispeels, 1983).

Lupinus angustifolius (lupin) seeds contain high levels of two storage proteins, conglutins α and β, which on the basis of both amino acid composition and M_r have been equated respectively with the legumin-like and vicilin-like proteins found in other legume seeds (Derbyshire *et al.*, 1976). Seeds of *L. angustifolius* contain, in addition, a third storage protein, conglutin γ, which differs markedly both in size and amino acid composition from either of these (Blagrove & Gillespie, 1975; Blagrove *et al.*, 1980). In the present paper we have studied the synthesis of conglutins α and γ in developing lupin seeds.

Storage proteins in other species are, in general, synthesized initially as families of precursors (Gatehouse *et al.*, 1984). In both *Pisum sativum* (pea) and *Glycine max* (soya bean) the precursors of the legumin-like proteins each contain two mature subunits linked as one continuous polypeptide (Tumer *et al.*, 1982; Croy *et al.*, 1980; Lycett *et al.*, 1984). The disulphide-bonded acidic and basic mature subunits are in each case produced by specific proteolytic cleavage of these precursors. In both *Pisum* and *Glycine*, cleavage of legumin and glycinin occurs within 2 h of synthesis (Chrispeels *et al.*, 1982*b*; Barton *et al.*, 1982). By contrast, in *L. angustifolius*, where it has been previously shown that the equivalent legumin-like protein, conglutin α, is also produced as a family of high-M_r precursors, cleavage of the precursors can be delayed for several days after synthesis (Gayler *et al.*, 1984).

In the present paper we have studied in detail both the synthesis of these relatively long-lived precursors of conglutin α and also the production from them of the subunits characteristic of the mature protein. Synthesis, secretion and proteolysis of conglutin α has, in addition, been compared with that for the minor storage protein conglutin γ, which at maturity is a protein that also contains two types of subunits linked in disulphide-bonded pairs (Blagrove *et al.*, 1980). Evidence has been obtained that the capacity for proteolysis of precursors of these different storage proteins in lupins differs markedly both between classes of the conglutins and between cotyledons at different stages of development.

Abbreviations used: DAF, days after flowering; poly(A)$^+$ RNA, polyadenylated RNA [i.e. RNA bound to oligo(dT)–cellulose]; SDS, sodium dodecyl sulphate.

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MATERIALS AND METHODS

Plant materials

Lupinus angustifolius (cv. Unicrop) was grown either in controlled-environment cabinets or under field conditions as previously described (Gayler *et al.*, 1984). Pods at mid-maturation (28–33 DAF) were harvested for isolation of polyribosomes and poly(A)$^+$ RNA. The seed coat and embryo were removed and the cotyledons instantaneously frozen in liquid N_2 under sterile conditions. Harvested cotyledons were stored at −70 °C before extraction of polyribosomes.

Labelling of cotyledons *in vivo*

L-[4,5-^{3}H]Leucine and [^{35}S]methionine were used for incorporation *in vivo* into newly synthesized proteins as described by Spencer *et al.* (1980). Freshly harvested cotyledons were placed on 10 μCi of radioisotope in a 20 μl droplet in a sterile humidified Petri dish. Cotyledons were then incubated under sterile conditions at room temperature and illuminated by fluorescent lights at 50 $\mu E \cdot s^{-1} \cdot m^{-2}$. Where required, cotyledons were rinsed and replaced on 20 μl of either sterile water or 1 mM-unlabelled amino acid for the duration of the 'chase'. After incubation, a 1 mm slice was taken from the face exposed to the radioisotope for extraction into 0.1 M-Tris/HCl buffer, pH 8.0, containing 10% (w/v) NaCl, 0.02% NaN_3 and 10 mM-2-mercaptoethanol at room temperature. The salt-soluble extract was dialysed into 60 mM-Tris/HCl buffer, pH 7.5, before further fractionation and analysis.

Immunoprecipitation

Antisera to conglutins α and γ were raised in rabbits and purified by affinity chromatography as previously described (Gayler *et al.*, 1984). Proteins were immunoprecipitated directly from the salt-soluble extract or total translation assays by using affinity-purified antibodies in 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.5 M-NaCl. Up to 30 μg of the appropriate unlabelled mature conglutin was added as carrier for immunoprecipitation from translation assays.

Isolation of polyribosomes and poly(A)$^+$ RNA

Membrane-bound polyribosomes were isolated from lupin cotyledons under sterile conditions as described by Matthews & Miflin (1980). Poly(A)$^+$ RNA was isolated from membrane-bound polyribosomes after dissociation in the presence of phenol. The polyribosome pellet was resuspended in 0.2 M-Tris/HCl buffer, pH 9.0, containing 0.1 M-NaCl, 0.01 M-EDTA, and 0.05% SDS and added to a 0.5 vol. of redistilled phenol saturated with the same buffer and heated for 5 min at 30 °C. Chloroform at half the initial resuspension volume was added and the aqueous and organic phases separated by centrifugation at 250 *g* for 5 min. Each phase was washed again and the total nucleic acids in the combined aqueous phase precipitated with 75% (w/v) ethanol and 0.4 M-ammonium acetate. The resulting pellet was dried under vacuum and resuspended in either sterile distilled water for storage or 10 mM-Tris/HCl buffer, pH 8.5, containing 0.4 M-NaCl, 1 mM-EDTA and 0.1% SDS for further fractionation. Chromatography of RNA on oligo(dT)–cellulose was performed as described by Bantle *et al.* (1976). Poly(A)$^+$ RNA was collected by precipitation with 75% (w/v) ethanol and 0.4 M-ammonium acetate, dried under vacuum, resuspended in sterile distilled water and stored at −70 °C.

Translation *in vitro*

A wheat-germ S30 cell-free extract was prepared as described by Roberts & Paterson (1973), the preincubation step being omitted. Translation assays were prepared as described by Matthews & Miflin (1980). Optimum conditions for incorporation of radioactivity into newly synthesized proteins were found with 0.3 mM-spermidine, 0.075 M-KCl, 1.6 mM-Mg^{2+} and either 5 μg of poly(A)$^+$ RNA or 20–40 μg of polyribosomes. Poly(A)$^+$ RNA was also translated in a commercial rabbit-reticulocyte-lysate system (The Radiochemical Centre, Amersham, Bucks., U.K.). Wheat-germ translation assays were centrifuged in a Beckman Airfuge at 132000 *g* for 30 min to pellet the ribosomes before immunoprecipitation of soluble proteins.

Endoplasmic-reticulum isolation and characterization

The isolation of endoplasmic reticulum and lysed protein bodies was adapted from the methods of Miflin *et al.* (1981) and Chrispeels *et al.* (1982*a*). Two isolation media were used; medium A contained 50 mM-Tricine/HCl buffer, pH 7.5, 100 mM-potassium acetate and 1 mM-EDTA, whereas in medium B EDTA was replaced with 10 mM-magnesium acetate. Slices from eight to ten radioactively labelled cotyledons were rinsed with distilled water and chopped with a razor blade in approx. 6 ml of 0.3 M-sucrose in the appropriate medium. This and all subsequent operations were carried out at 4 °C. The homogenate was squeezed through Miracloth and centrifuged at 1000 *g* to remove nuclei, chloroplasts, starch and cell debris. The supernatant was then layered on to continuous 16–55%-(w/v)-sucrose-density gradients constructed in the same medium as that used for homogenization. Gradients were centrifuged in a Beckman SW28 rotor for 2.5 h at 82700 *g* ($r_{av.}$ 12.2 cm), then fractionated by using an ISCO density-gradient fractionator. Fractions were analysed for marker-enzyme activity and trichloroacetic acid-precipitable radioactivity. Peak fractions were immunoprecipitated with the appropriate sera. Assays for NADH: cytochrome *c* reductase were performed as described by Bowles & Kauss (1976), except that 1.6 mM-NaN_3 replaced NaCN. Cytochrome *c* oxidase and inosine diphosphatase were assayed by the method of Bollini & Chrispeels (1979) and Chrispeels (1983) to identify the positions of mitochondrial and Golgi membranes respectively.

Gel electrophoresis

Electrophoresis was performed by using linear polyacrylamide gradient gels [8–20% (w/v) polyacrylamide] under reducing and dissociating conditions essentially as described by Spencer *et al.* (1980). Radioactively labelled polypeptides were identified by fluorography of the fixed and stained gel (Gayler & Sykes, 1981; Laskey & Mills, 1975).

RESULTS

Synthesis of conglutin *α in vivo* and in cell-free systems

[^{3}H]Leucine was used *in vivo* to label proteins in isolated lupin cotyledons. In previous work it had been shown that when cotyledons were labelled *in vivo* for only 2 h, each of the three precursor polypeptides of conglutin

α became labelled, but no breakdown products equivalent to the processed subunits were found (Gayler *et al.*, 1984). As shown in Fig. 1 (track 6), when cotyledons from pods aged up to 28 DAF were incubated for much longer periods (16–24 h) with [^{3}H]leucine, again only three major polypeptides with apparent M_r values 85000, 72000 and 64000 could be immunoprecipitated by affinity-purified anti-(conglutin α) sera. Again, no breakdown products were found, even after 16 h labelling. These peptides were identical with those identified by Western blotting, which accumulate in cotyledons up to 33 DAF and were previously estimated to have apparent M_r values 83000, 68000 and 62000 (Gayler *et al.*, 1984). Long-term (16–24 h) radioactively labelled peptides were therefore used for comparison with other synthetic products throughout the present study.

The production of conglutin α in cell-free systems was studied in two ways. Products labelled *in vivo* were first compared with those produced from an initiating system using poly(A)$^+$ RNA to produce completely unmodified peptides.

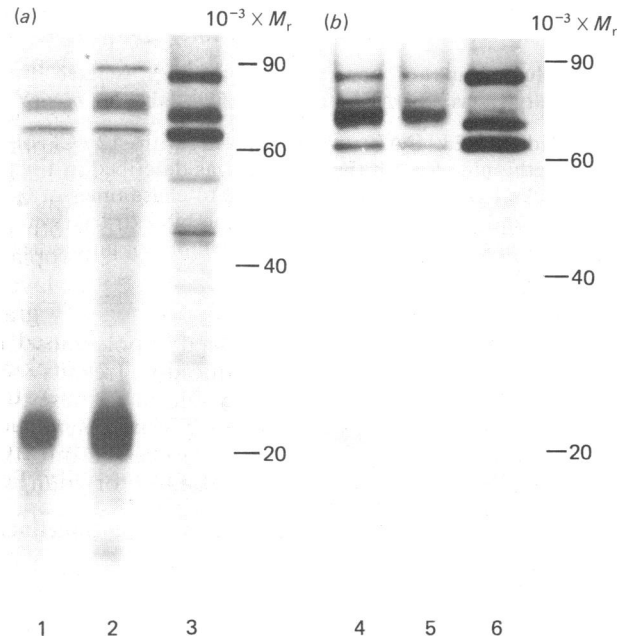

Fig. 1. Fluorographs comparing conglutin α-related polypeptides produced *in vitro* from cell-free translations and *in vivo* from labelled cotyledons

Polypeptides labelled with [^{3}H]leucine and immunoprecipitated with anti-(conglutin α) antibodies were obtained from cell-free translations containing either (*a*) 5 μg of poly(A)$^+$ RNA isolated from mid-maturation cotyledons (tracks 1 and 2) or (*b*) 20 μg of membrane-bound polyribosomes from mid-maturation cotyledons (tracks 4 and 5) using either S30 wheat-germ (tracks 1, 4 and 5) or rabbit reticulocyte lysate (track 2). The electrophoretic mobilities of these polypeptides are compared with those of conglutin α polypeptides labelled for 16 h *in vivo* in cotyledons aged either (*a*) between 28 DAF and 31 DAF (track 3) or (*b*) 28 DAF (track 6) as described in Fig. 2. Label observed at $M_r \sim 22000$ in translation tracks is a non-specific contaminant of all such immunoprecipitations. The scale of apparent M_r used in all Figures was determined with standard proteins.

Poly(A)$^+$ RNA was isolated from membrane-bound polyribosomes and used to direct synthesis of the lupin storage proteins *in vitro* in both the wheat-germ and rabbit-reticulocyte-lysate translation systems. Translation of poly(A)$^+$ RNA from mid-maturation cotyledons in both systems produced a family of polypeptides, related to conglutin α, which were identified by immunoprecipitation from the translation mix after the ribosomal fraction had been removed. These primary translation products were shown on SDS/polyacrylamide gels to have apparent-M_r values higher than those produced by labelling *in vivo* (Fig. 1*a*, tracks 1 and 2), and no products equivalent in size to the small subunits characteristic of mature conglutin α were found.

Such higher apparent-M_r values as were observed for these primary translation products (68000–88000) would be expected if the proteins were initially produced with a leader sequence similar to that in other secretory proteins (Von Heijne, 1983). Consistent with this were the results obtained when membrane-bound polyribosomes were purified from cotyledons and added to the wheat-germ translation system to allow chain completion to occur. In this second type of translation the polypeptides should have been produced without the leader sequence. In fact, as shown in Fig. 1(*b*) (tracks 4 and 5), the polypeptides produced in such translations, which were immunologically related to conglutin α, did include those with apparent-M_r values of 64000 and 85000; that is, apparent-M_r values identical with those of two of the peptides of conglutin α labelled *in vivo*. By contrast, neither of the polypeptides with apparent-M_r values of 68000 and 88000 detected in poly(A)$^+$ RNA primed translations were produced from the polyribosome. These differences in M_r values suggest that co-translational cleavage removed 30–40 amino acids ($M_r \sim 3000$–4000) from the primary transcripts of conglutin α, before their accumulation as either polyribosome products or products labelled *in vivo*.

Such a conclusion is only clear for the largest and the smallest precursors. Similar determination of the relationship between the polypeptides with M_r values of approx. 70000–75000, which can also be detected in translations directed by either poly(A)$^+$ RNA (Fig. 1*a*) or polyribosomes (Fig. 1*b*), and the polypeptides labelled *in vivo* is not possible because of the multiplicity of polypeptides observed in this size range. Nevertheless, this diversity suggests that conglutin α probably arises from a multigene family equivalent to those characteristic of other storage-protein genes (Higgins, 1984).

Processing of pre-(conglutin α) to mature conglutin α

The source of the lower-M_r peptides of conglutin α, which begin accumulating in developing lupin cotyledons 33 DAF (Gayler *et al.*, 1984), was investigated by pulse–chase experiments in intact cotyledons of this age. A 1 h labelling period followed by up to 8 h of incubation without isotopic labelling demonstrated that the same precursor peptides found in younger seeds were being synthesized in the older cotyledons (Fig. 2). However, after a 24 h chase, peptides with estimated M_r values of 62000, 49000, 42000 and a group from 21000 to 24000 appeared and the precursors disappeared (Fig. 2). Equivalent results were obtained in these pulse–chase experiments when either unlabelled 1 mM-leucine or distilled water was used during the 'chase' period. The measured M_r values for the breakdown products

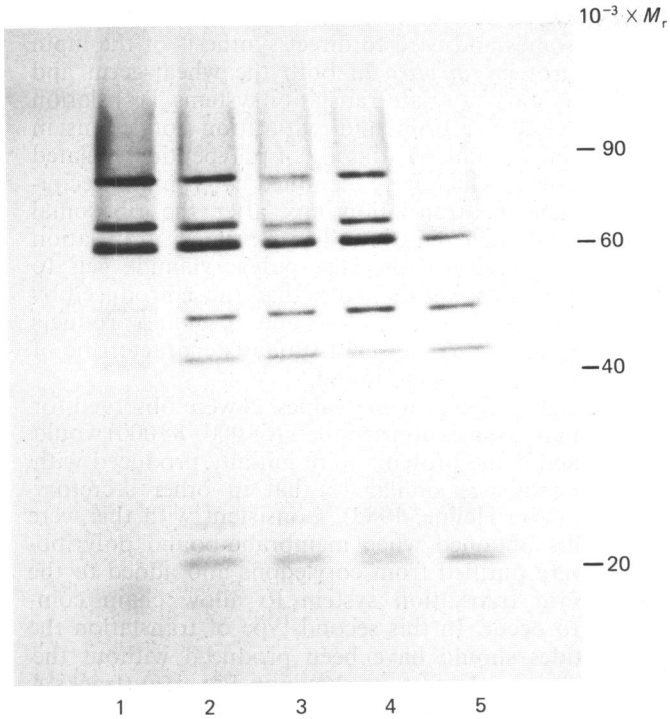

Fig. 2. Fluorograph of conglutin α polypeptides isolated from cotyledons 33 DAF after pulse–chase labelling *in vivo*

Cotyledons 33 DAF were labelled *in vivo* with 10 μCi of [^{3}H]leucine for 1 h, then incubated on sterile distilled water for 0, 2, 4, 8 and 24 h (tracks 1, 2, 3, 4 and 5 respectively). Total globulins were extracted into 0.1M-Tris/HCl buffer, pH 8.0, containing 10% (w/v) NaCl, 0.02% NaN_3 and 10 mM-2-mercaptoethanol. Particular conglutins were immunoprecipitated with antibodies raised against conglutin α and separated by electrophoresis on polyacrylamide gels in the presence of SDS after reduction with 2-mercaptoethanol and detected by fluorography.

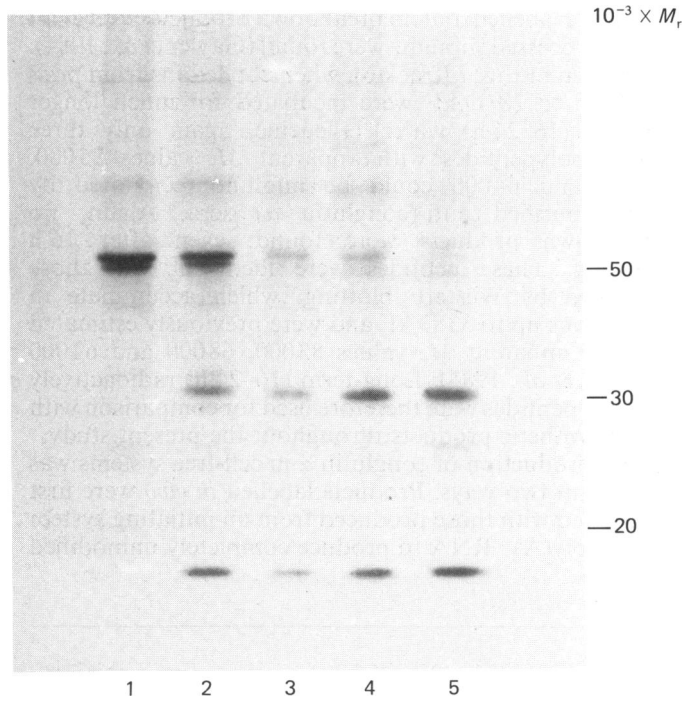

Fig. 3. Fluorograph of conglutin polypeptides isolated from cotyledons 28 DAF after pulse–chase labelling *in vivo*

Cotyledons (28 DAF) were labelled with 12 μCi of [^{35}S]methionine for 1 h then 'chased' as described in Fig. 2. Tracks 1, 2, 3, 4 and 5 correspond to chase times of 0, 2, 4, 8 and 24 h respectively. Salt-soluble extracts were immunoprecipitated with anti-(conglutin γ) antibodies before electrophoresis.

correspond to those peptides which accumulate in cotyledons between 33 and 50 DAF (Gayler *et al.*, 1984) and were the only polypeptides previously detected with anti-(conglutin α) serum in Western-blot analyses of total globulin extracts 50 DAF. There is, therefore, a direct precursor–product relationship between the peptides accumulating in juvenile cotyledons and the major subunits observed in seeds approaching maturity. Cleavage of the precursors can, however, only be demonstrated in tissue aged 33 DAF or older. Equivalent labelling studies in juvenile tissue aged 28 DAF or younger produced only the major precursors (Fig. 1).

Synthesis and processing of conglutin γ in juvenile cotyledons

The synthesis of conglutin α in lupins was compared with that of the more simple storage protein, conglutin γ. Unlike conglutin α, conglutin γ accumulates in cotyledons throughout development in its mature form as two peptides of apparent M_r 32000 and 17000 (Gayler *et al.*, 1984). However, labelling *in vivo* with [^{35}S]methionine demonstrated that this protein is synthesized as short-lived higher-M_r precursors. The major precursor labelled with [^{35}S]methionine in 2 h in juvenile cotyledons has an apparent M_r of 51000, and there is a minor polypeptide with an apparent M_r of 50000 (Fig. 3). Peptides of similar M_r are produced in polyribosome run-off experiments with [^{35}S]methionine (results not shown). Unlike the precursors of conglutin α, these two peptides have half-lives of less than 2 h after synthesis and, as also shown in Fig. 3, can be processed into the mature peptides even in tissue aged 28 DAF or younger.

Isolation of storage proteins during intracellular transport

Since in juvenile cotyledons there were major differences in the capacities of tissue to process the precursors of conglutin α and conglutin γ to mature forms, it was necessary to establish whether secretion of these two proteins was normal in both cases. Subcellular organelles were isolated on continuous sucrose density gradients. Endoplasmic reticulum was located by assaying for the marker enzyme antimycin-insensitive NADH:cytochrome *c* reductase. Its identity was also confirmed by observing the shift in banding density when the ribosomes were detached from the membranes in the absence of Mg^{2+} (Miflin *et al.*, 1981).

A single NADH:cytochrome *c* reductase activity peak with a density of 1.13 $g \cdot cm^{-3}$ was detected in the absence of Mg^{2+}, whereas in its presence the band shifted to a density of 1.18 $g \cdot cm^{-3}$. These values are consistent with the shift in density reported for endoplasmic reticulum isolated from pea cotyledons (Miflin *et al.*, 1981; Chrispeels *et al.*, 1982*a*). As expected, cytochrome *c* oxidase activity remained at a density of 1.18 $g \cdot cm^{-3}$ with either treatment (Miflin *et al.*, 1981; Higgins *et al.*, 1983). Where measured, IDPase activity coincided with

NADH:cytochrome *c* reductase activity, indicating the possible contamination of the endoplasmic-reticulum fraction with Golgi-like vesicles. Fractions showing peak NADH:cytochrome *c* reductase in the absence of Mg^{2+} were selected for immunological fractionation of storage proteins.

Protein bodies of pea cotyledons have been reported to lyse when prepared in the absence of Mg^{2+} by the procedure used in the present paper (Chrispeels *et al.*, 1982*a*), and more than 95% of the reserve proteins of peas were found in the sample volume of such gradients. A similar proportion of the storage proteins of the lupin were found in the sample volume of equivalent gradients when protein bodies from lupins were prepared in the absence of Mg^{2+}, indicating a similarly extensive lysis of those protein bodies had also occurred. Direct isolation of protein bodies on discontinuous gradients confirmed their extensive lysis in the absence of Mg^{2+}. It was concluded that proteins present *in vivo* in either the cytosol or in protein bodies were to be found in the sample volume of these gradients, well separated from those in the endoplasmic reticulum.

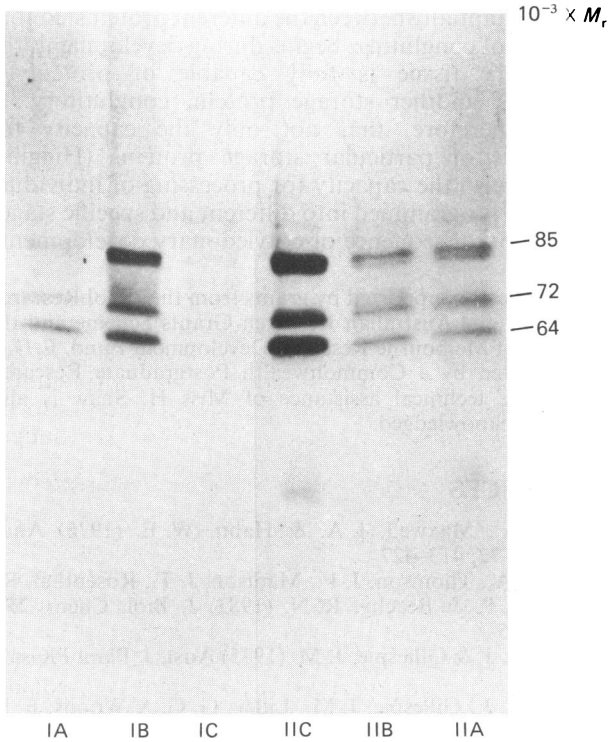

Fig. 4. Fluorograph showing sequestration and transport of conglutin α precursors through the endoplasmic-reticulum fraction of cotyledons 28 DAF

Cotyledons 28 DAF were labelled *in vivo* with [^{3}H]leucine for 1.5 h (tracks IA and IIA), 3 h (tracks IB and IIB) and 3 h + 21 h 'chase' (tracks IC and IIC) as described in Fig. 2. Endoplasmic reticulum (microsomal membranes) (I) was separated from cytosolic proteins and lysed protein bodies (soluble fraction) (II) on 16–55% (w/v)-sucrose density gradients. Conglutin α polypeptides were recovered from each fraction by immunoprecipitation with anti-(conglutin α) antibodies and separated on SDS/polyacrylamide-gel electrophoresis as in Fig. 2. M_r values are shown for each of the major conglutin α precursors.

Transport of conglutin α through the endoplasmic reticulum

Labelled proteins extracted from cotyledons that were aged 28 DAF and had been incubated with [^{3}H]leucine before organelle fractionation on density gradients are shown in Fig. 4. Labelled peptides immunologically related to conglutin α were found in both the sample volume and the endoplasmic-reticulum fraction. As expected, the only polypeptides found had the same apparent M_r values as the juvenile precursors which accumulate in cotyledons of the same age and are labelled *in vivo* in such tissue (Fig. 1*b*, track 6). No smaller fragments could be detected (Fig. 4).

As is also shown in Fig. 4, further transport of the precursors of conglutin α out of the endoplasmic reticulum could be monitored by pulse–chase labelling. Intact cotyledons were pulse-labelled with [^{3}H]leucine for 1–3 h and subsequently incubated with distilled water for the duration of the chase. During the chase period, all three labelled conglutin α precursors completely moved out of the endoplasmic reticulum during the chase, indicating that delay in further secretion of the precursors sequestered into the endoplasmic reticulum was not responsible for the delay in processing of conglutin α observed in juvenile tissue. Equivalent studies with [^{35}S]methionine-labelled cotyledons confirmed that conglutin γ was also sequestered in precursor form (M_r 51000) into the endoplasmic reticulum. Conglutin γ also disappeared from the endoplasmic reticulum fraction within 2 h when pulse–chased.

DISCUSSION

Co-translational cleavage of primary products is characteristic of many exported proteins (Von Heijne, 1983; Higgins, 1984). The signal-sequence hypothesis (Walter & Blobel, 1981) predicts that exported proteins are directed to the lumen of the endoplasmic reticulum with the concomitant removal of the leader peptide in the early stages of synthesis. Signal (leader) sequences from eukaryotic systems are characteristically 15–30 amino acids (M_r 1500–3000) in length (Von Heijne, 1983). The presence of leader sequences on seed-storage-protein translation products has been demonstrated by observing co-translational cleavage (Bollini *et al.*, 1983; Higgins & Spencer, 1981; Tumer *et al.*, 1982) and by sequencing of cDNA and genomic clones of storage-protein genes (Lycett *et al.*, 1984). Since the apparent-M_r values for the precursors of conglutin α produced in cell-free systems primed by poly(A)$^+$ RNA are 3000–4000-M_r larger than their respective counterparts *in vivo*, it is likely that the conglutin α precursors are also produced with attached leader sequences. Translation of conglutin α, as described here, therefore conforms to the model of secretory-protein synthesis.

Pea- and soya-bean-protein genes have been found to occur as families probably arising from gene duplication (Lycett *et al.*, 1983; Higgins, 1984; Schuler *et al.*, 1982). The present results suggest that the three major precursors of conglutin α synthesized *in vivo* are likewise derived from individual mRNA species. The multiplicity of minor pre-conglutin α precursors with M_r values between 75000 and 78000, which are observed in cell-free translation studies (Fig. 1), presumably also reflects the expression of such multigene families.

Earlier studies in this laboratory showed that the nature of accumulated storage proteins changed radically at the mid-point of cotyledonary development (Gayler *et al.*, 1984). The results presented here demonstrate that mRNA from cotyledons at mid-stage development does not code for the lower-M_r fragments of conglutin α that apear at this stage. Rather it is suggested that such fragments are produced by internal proteolysis of the high-M_r precursors.

A model is proposed in Fig. 5 suggesting that the conglutin α peptides of M_r less than 50000 generated during pulse–chase studies (Fig. 2) could in fact arise by proteolysis of a single common cleavage site in the precursors. We propose that *C*-terminal peptides with apparent M_r values of 22000–23000 are cleaved from each of the precursors that accumulate in the protein bodies. The peptides shown in this model correspond in size both to those identified by the pulse–chase labelling *in vivo* of cotyledons aged 33 DAF (Fig. 2) and also to those identified as subunits of mature conglutin α under reducing conditions (Blagrove & Gillespie, 1975). This model predicts sequence homology between precursors of conglutin α at least around the cleavage site. This is still to be tested. The precursor–product relationship thus demonstrated in the labelling experiments therefore parallels the production of the acidic and basic paired subunits of glycinin from similar precursors in soya beans (Tumer *et al.*, 1981) and of legumin in peas (Croy *et al.*, 1980). Conglutin α therefore appears to have an equivalent path of biosynthesis to these and other 11S seed storage proteins (Higgins, 1984).

The precursor peptides of conglutin α that accumulate in protein bodies after 33 DAF are rapidly cleaved, but in seeds of younger age no such processing occurs. Normally storage proteins move through the endoplasmic reticulum immediately after synthesis and then are transported to the protein bodies. This has been reported for peas (Chrispeels *et al.*, 1982*a*) and beans (*Phaseolus* sp.) (Bollini & Chrispeels, 1979). In peas, proteolytic processing of pea legumin and vicilin precursors is confined to the protein bodies, although the endoplasmic reticulum is the site of assembly of mature-sized oligomers (Chrispeels *et al.*, 1982*b*).

If transport in lupin cotyledons < 33 DAF was disturbed, storage proteins may have been confined to the endoplasmic reticulum without exposure to proteolytic enzymes. Certainly, organelle fractionation of radioactively labelled cotyledons < 33 DAF demonstrated that the precursors for both conglutin α and γ were sequestered in the endoplasmic reticulum after synthesis. However, it was also shown that both these sets of radioactive precursors could also be 'chased' out of the endoplasmic reticulum fraction within hours of synthesis. Therefore it was concluded that transport of conglutins α and γ both occurred within hours of synthesis in juvenile tissue in the same manner as pea legumin and vicilin (Chrispeels *et al.*, 1982*a*). Since conglutin γ was quickly cleaved into its mature subunits at a rate similar to that for peak legumin, even in juvenile tissue, but conglutin α was not, it was concluded that juvenile cotyledons simply did not have the capacity to process conglutin α to its mature subunits.

The enzyme responsible for this cleavage must therefore be inactive in protein bodies of lupin seeds aged < 28 DAF. Whether the enzymic activity detected in more mature protein bodies from seeds aged > 33 DAF is the result of activation of pre-existing enzyme or the result of synthesis *de novo* and secretion of new enzymes into the protein bodies is still to be established. Nevertheless, it is clear that the time at which developing cotyledonary tissue of lupins acquires the capacity for the proteolytic processing of the subunits of storage proteins differs substantially between the different proteins. Before processing of conglutin α begins during development, the cotyledonary tissue is fully capable of proteolytic cleavage of another storage protein, conglutin γ. It appears, therefore, that not only the capacity for biosynthesis of particular storage proteins (Higgins, 1984), but also the capacity for processing of individual proteins, is programmed into different and specific stages in the temporal sequence of cotyledonary development.

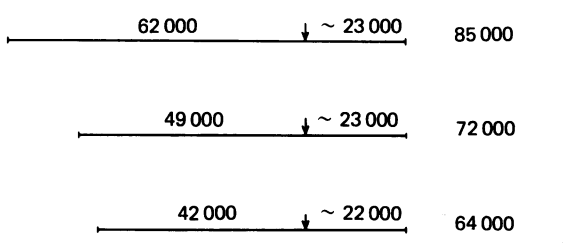

Fig. 5. Model depicting the relationship between the subunits of conglutin α and its precursors

Apparent-M_r values are shown for each of the precursors of conglutin α (85000, 72000 and 64000) and the postulated positions within these of each of the products (62000, 49000, 42000 and ~ 23000) produced from these precursors by pulse labelling as in Fig. 2. ↓ indicates the site of the postulated common cleavage site in each precursor.

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REFERENCES

Bantle, J. A., Maxwell, I. A. & Hahn, W. E. (1976) Anal. Biochem. **72**, 413–427

Barton, K. A., Thomson, J. F., Madison, J. T., Rosenthal, R., Jarvis, N. P. & Beachy, R. N. (1982) J. Biol. Chem. **257**, 6089–6095

Blagrove, R. J. & Gillespie, J. M. (1975) Aust. J. Plant Physiol. **2**, 13–27

Blagrove, R. J., Gillespie, J. M., Lilley, G. G. & Woods, E. F. (1980) Aust. J. Plant Physiol. **7**, 1–13

Bollini, R. & Chrispeels, M. J. (1979) Planta **146**, 487–501

Bollini, R., Vitale, A. & Chrispeels, M. J. (1983) J. Cell. Biol. **96**, 999–1007

Bowles, D. J. & Kauss, H. (1976) Biochim. Biophys. Acta **443**, 360–374

Chrispeels, M. J. (1983) Planta **158**, 140–151

Chrispeels, M. J., Higgins, T. J. V., Craig, S. & Spencer, D. (1982*a*) J. Cell. Biol. **93**, 5–14

Chrispeels, M. J., Higgins, T. J. V. & Spencer, D. (1982*b*) J. Cell. Biol. **93**, 306–313

Croy, R. R. D., Gatehouse, J. A., Evans, I. A. & Boulter, D. (1980) Planta **148**, 49–56

Derbyshire, E., Wright, D. J. & Boulter, D. (1976) Phytochemistry **15**, 3–24

Gatehouse, J. A., Croy, R. R. D. & Boulter, D. (1984) Crit. Rev. Plant Sci. **1**, 287–314

Gayler, K. R. & Sykes, G. E. (1981) Plant Physiol. **67**, 958–961
Gayler, K. R., Boadle, B. G., Snook, M. & Johnson, E. D. (1984) Biochem. J. **221**, 333–341
Higgins, T. J. V. (1984) Annu. Rev. Plant Physiol. **35**, 191–221
Higgins, T. J. V. & Spencer, D. (1981) Plant Physiol. **67**, 205–211
Higgins, T. J. V., Chrispeels, M. J., Chandler, P. M. & Spencer, D. (1983) J. Biol. Chem. **258**, 9550–9552
Laskey, R. A. & Mills, A. D. (1975) Eur. J. Biochem. **56**, 335–341
Lycett, G. W., Delauney, A. J., Gatehouse, J. A., Gilroy, J., Croy, R. R. D. & Boulter, D. (1983) Nucleic Acids Res. **11**, 2367–2380
Lycett, G. W., Croy, R. R. D., Shirsat, A. H. & Boulter, D. (1984) Nucleic Acids Res. **12**, 4493–4506
Matthews, J. A. & Miflin, B. J. (1980) Planta **149**, 262–268
Miflin, B. J., Burgess, S. R. & Shewry, P. R. (1981) J. Exp. Bot. **32**, 199–219
Roberts, B. E. & Paterson, B. M. (1973) Proc. Natl. Acad. Sci. U.S.A. **70**, 2330–2334
Schuler, M. A., Schmitt, E. S. & Beachy, R. N. (1982) Nucleic Acids Res. **10**, 8225–8243
Spencer, D., Higgins, T. J. V., Button, S. C. & Davey, R. A. (1980) Plant Physiol. **66**, 510–515
Tumer, N. E., Thanh, V. H. & Nielsen, N. C. (1981) J. Biol. Chem. **256**, 8756–8760
Tumer, N. E., Richter, J. D. & Nielsen, N. C. (1982) J. Biol. Chem. **257**, 4016–4018
Von Heijne, G. (1983) Eur. J. Biochem. **133**, 17–21
Walter, P. & Blobel, G. (1981) J. Cell. Biol. **91**, 557–561

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